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(54) **A process for producing an aqueous solution of sodium hyaluronate**

(57) **A process for producing an aqueous solution of sodium hyaluronate having high purity, which process comprises treating a fermentation broth of hyaluronic acid with active carbon in the presence of sodium chloride, optionally after having treated the broth by ultrafiltration, and then treating the active carbon-treated broth by ultrafiltration.**

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A PROCESS FOR PRODUCING AN AQUEOUS SOLUTION
OF SODIUM HYALURONATE

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The present invention relates to a process for producing an aqueous solution of sodium hyaluronate having high purity from a fermentation broth of hyaluronic acid.

10 Hyaluronic acid is found as a constituent ingredient in connective tissues, vitreous humor, umbilical cords, cartilage, skins, rooster combs and the like, and it plays an important role in a living body. Sodium hyaluronate is a high-molecular substance. As its solution has
15 high viscosity, elasticity and water-holding property, it is widely used in cosmetic materials. It is also used in medicines for ophthalmopathy, wounds and arthropathy.

Sodium hyaluronate used for the above purposes is required to have high molecular weight and high purity.

20 Sodium hyaluronate is industrially produced by extracting from rooster combs, or by incubating microorganism having the ability to produce hyaluronic acid in a culture medium (a fermentation method).

Hitherto, an aqueous solution of highly pure sodium
25 hyaluronate has been produced by a process comprising treating a solution containing hyaluronic acid, which is obtained by extraction or fermentation, with a quaternary ammonium salt to obtain a precipitate, redissolving the precipitate in water, treating the solution with protease
30 and with active carbon, adding alcohol to the treated solution to precipitate sodium hyaluronate, separating the precipitate from the alcohol solution, drying the precipitate to obtain a sodium hyaluronate powder, and redissolving the powder in water.

35 In such a process, it needs troublesome methods after

a culture medium is purified, a precipitating agent such as ethanol is added to the purified solution to precipitate sodium hyaluronate powder, and the powder is redissolved in water to obtain an aqueous solution of highly pure sodium hyaluronate.

An aqueous solution of highly pure sodium hyaluronate is not produced without precipitation process from a fermentation broth of hyaluronic acid by conventional methods.

An object of the present invention is to provide a process for directly producing an aqueous solution of highly pure sodium hyaluronate from a fermentation broth without using the above mentioned troublesome methods,

The present invention is characterized in that a fermentation broth of hyaluronic acid is treated with active carbon, the treated solution is filtered, and the filtrate is treated by ultrafiltration to obtain an aqueous solution of highly pure sodium hyaluronate which can be used in cosmetic materials.

The term of "highly pure" in the present invention has the following meaning. The purity of sodium hyaluronate is at least 85% by weight of freeze-dried material which is obtained by freeze-drying an aqueous solution of sodium hyaluronate, the evaporation residue of the solution is 100 to 130% by weight of sodium hyaluronate and protein in the solution is less than 0.1% by weight of sodium hyaluronate.

Sodium hyaluronate used in the present invention can be obtained from a culture medium in which a microorganism having the ability to produce hyaluronic acid is incubated in that culture medium. In the present invention, microorganisms which have the ability to produce hyaluronic acid can be used, for example, Genus Streptococcus is preferred. Streptococcus pyogenes, Streptococ-

cus equi, Streptococcus equisimilis, Streptococcus dysgalactial and Streptococcus zooepidemicus are exemplified.

A culture medium used in the present invention can be a conventional culture medium which is used for incubation of a hyaluronic acid producing microorganisms. As an example, a culture medium containing 2.0 to 3.0% of dextrose, 0.5% of yeast extract, 0.3% of potassium dihydrogen phosphate, 0.2% of dipotassium phosphate, 0.01% of sodium thiosulfate, 0.01% of magnesium sulfate $7H_2O$, 0.002% of sodium sulfite, 0.001% of cobalt chloride, 0.001% of manganese chloride and 0.5% of an antifoamer can be used at pH 6.0 to 8.5 (in the solution, % means g/dl). The incubation is conducted by shaking or aerating under aerobic conditions. The incubation temperature is 25 to 40°C and preferably 30 to 35°C. The pH value is controlled at 6.5 to 8.0 and preferably 7.0, after 1 to 3 days of the usual incubation periods, hyaluronic acid is accumulated in the culture medium. The culture medium is a mixture of culture residue ingredients, high-molecular ingredients, low-molecular ingredients, coloring matters, microorganisms and hyaluronic acid.

High-molecular ingredients excepting hyaluronic acid, coloring matters and a part of low-molecular ingredients are removed by active carbon adsorption. In particular, it is essential that protein of high-molecular ingredients, by which an allergy reaction is caused, is thoroughly removed. The culture medium is treated with active carbon, and the active carbon and the microorganisms are removed by filtration.

The present inventors investigated conditions for adsorbing and removing the high-molecular ingredients, particularly protein by using active carbon. As a result, they found that the greater part of the protein could be adsorbed and removed by treating with active carbon in the presence of at least 0.2M of sodium chloride. The

relation between the concentration of sodium chloride in the treatment with active carbon and the protein content of the obtained sodium hyaluronate is shown in Table 1.

Table 1

5	Concentration of sodium chloride in active carbon treatment (M)	Protein content per sodium hyaluronate (% by weight)
10	0	0.93
	0.01	0.67
	0.05	0.32
	0.1	0.16
	0.2	0.09
15	0.3	0.05
	0.4	0.04

As shown in Table 1, it is necessary to treat with active carbon in the presence of at least 0.2M, preferably 0.3 to 0.4M of sodium chloride. The filtrate which has been treated with active carbon can be subjected to ultrafiltration to remove the residual low molecular ingredients which are derived from the culture medium or produced in the fermentation process and sodium chloride which is added in the active carbon treatment.

Hyaluronic acid in the fermentation broth has commonly a molecular weight of at least 7 to 8 hundred thousands. On the other hand, dextrose and mineral salts which are used as fermentation raw materials and a salt of organic acids which is produced in the fermentation period have commonly a molecular weight of less than 5 thousands.

Accordingly, when the ultrafiltration treatment is conducted with an ultrafiltration membrane which is able

to cut compounds having a molecular weight of 6000 to 50000 or less, the above mentioned low molecular ingredients can be removed. However, since low molecular ingredients cannot be removed satisfactorily by only one pass
5 ultrafiltration operation, it is necessary to repeat the ultrafiltration operation by adding purified water intermittently or continuously. With the low molecular ingredients and sodium chloride are removed, the electric conductivity of the filtrate is lowered. Accordingly, the
10 purification progress is judged by determination of the electric conductivity. For example, purified water is added to a filtrate obtained by active carbon treatment of the fermentation broth of hyaluronic acid. The mixture is subjected to ultrafiltration with Minitan (cut-off
15 molecular weight: 30000) manufactured by Nihon Millipore Co. Ltd, and the relationships among the electric conductivity of the mixture, the purity of sodium hyaluronate and the amount of evaporated residue are shown in Table 2. The value of the electric conductivity changes with
20 the concentration of sodium hyaluronate, so that the former is shown in a case of 0.2% by weight (at 25°C) of the latter.

Table 2

25	Electric conductivity	Purity*1	Evaporated residue*2
	(mS/cm)	(% by weight)	(% by weight)
30	20.0	6.0	17.5
	5.8	17.8	6.6
	3.6	36.7	3.2
	0.50	79.8	1.50
	0.37	89.1	1.25
	0.20	91.3	1.11
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*1 Purity of sodium hyaluronate per freeze-dried material of sodium hyaluronate solution

*2 Value calculated in terms of 1% of sodium hyaluronate

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As a result, to prepare a highly pure sodium hyaluronate solution, it is necessary to treat the solution by ultrafiltration until the value of the electric conductivity lowers to less than 0.4 mS/cm.

Further, in the process of the present invention, after the solution is treated with active carbon and the active carbon is separated and removed from the solution, the ultrafiltration treatment is conducted. Otherwise, the solution can be treated by ultrafiltration, active carbon treatment, separation and removal of the active carbon, and ultrafiltration. In this case, a liquid suited for the first ultrafiltration is a liquid obtained by filtration to remove microorganisms from the fermentation broth. Before the solution is treated with active carbon, the solution is treated by ultrafiltration. Accordingly, in comparison with a case in which the solution is not treated by ultrafiltration before the active carbon treatment, when the solution is treated by ultrafiltration before the active carbon treatment, the same effect is obtained with a small amount of active carbon.

By using the present invention, an aqueous solution of sodium hyaluronate having high purity can be prepared directly from a fermentation broth. Further, considering the use application, the solution can be finally concentrated with a filter or a concentrator by a conventional method.

By using the present invention, an aqueous solution of sodium hyaluronate having high purity can be prepared directly from a fermentation broth without precipitation process. The method of the present invention provides a simple process without using conventional troublesome

methods. The cost of the process is lowered. It becomes possible to obtain a highly pure product usable as cosmetic materials, medicines, etc..

- 5 The following examples illustrate the present invention more specifically, but these will not always be precise in practical applications.

Example 1

- 300 ml of a fermentation broth of hyaluronic acid was
10 diluted three times with water, sodium chloride was added to the solution to obtain a solution in concentration of 0.3M sodium chloride. 60g of active carbon (manufactured by Takeda Seiyaku Kogyo Co. Ltd, in Japan, trade name: Shirasagi A-50W, containing water of 50% by weight) was
15 added to the solution, the solution was stirred for one hour and the active carbon was filtered off. The electric conductivity of the solution was 22.0 mS/cm. The filtrate was subjected to ultrafiltration with an ultrafiltration membrane (manufactured by Nihon Millipore Co. Ltd., trade
20 name: Minitan) having a cut-off molecular weight of 30000 by using purified water to obtain a treated solution having an electric conductivity of 0.24 mS/cm. The protein content of the treated solution was 0.06% by weight of sodium hyaluronate, the residue on evaporation was
25 115% by weight of sodium hyaluronate and the purity of sodium hyaluronate was 90.4% by weight of the freeze-dried material. A solution of sodium hyaluronate having high purity which was suited for cosmetics was obtained.

Comparative Example 1

- 30 Using the same fermentation broth as used in Example 1, ultrafiltration was conducted except that active carbon was not added, and a filtrate having an electric conductivity of 0.30 mS/cm was obtained. The protein content of the treated solution was 2.74% by weight of
35 sodium hyaluronate, the residue on evaporation was 125%

by weight of sodium hyaluronate and the purity of sodium hyaluronate was 85.5% by weight of the freeze-dried materials. Accordingly, the removal of protein from the solution was incomplete.

5 Comparative Example 2

Using the same fermentation broth as used in Example 1, ultrafiltration was conducted except that sodium chloride was not added, and a filtrate having an electric conductivity of 0.20 mS/cm was obtained.

10 The protein content of the treated solution was 1.12% by weight of sodium hyaluronate, the residue on evaporation was 120% by weight of sodium hyaluronate and the purity of sodium hyaluronate was 86.3% by weight of the freeze-dried materials. Accordingly, the removal of
15 protein from the solution was incomplete.

Example 2

300 ml of a fermentation broth of hyaluronic acid was diluted three times with water, sodium chloride was added to the solution to obtain a solution in concentration of
20 0.4M sodium chloride. The electric conductivity of the solution was 28.3 mS/cm. After filtration for removing microorganisms from the solution, the filtrate was subjected to ultrafiltration with an ultrafiltration membrane having a cut-off molecular weight of 6000 (manufactured by Asahi Kasei Co. Ltd. in Japan, ultrafiltration
25 module: SIP-1013) by using purified water to obtain a treated solution having an electric conductivity of 0.38 mS/cm. Sodium chloride was added to the treated solution to obtain a solution of 0.4M sodium chloride. 30g of
30 active carbon (manufactured by Hutamura Kagaku Co. Ltd. in Japan, trade name: Taiko S, water content: 50% by weight) was added to the solution, the solution was stirred for one hour and the active carbon was filtered off.

35 The filtrate obtained by the filtration of the active

carbon was subjected to ultrafiltration with the same ultrafiltration membrane as mentioned above by adding purified water, and a filtrate having an electric conductivity of 0.2 mS/cm was obtained.

- 5 The protein content of the treated 0.2% aqueous solution of sodium hyaluronate was 0.04% by weight of sodium hyaluronate, the residue on evaporation was 114% by weight of sodium hyaluronate and the purity of sodium hyaluronate was 92.2% by weight of the freeze-dried
10 material. The 0.2% aqueous solution was subjected to ultrafiltration with the same ultrafiltration membrane, and a 1% aqueous solution of sodium hyaluronate was easily prepared.

Comparative Example 3

- 15 Using the same fermentation broth as used in Example 2, ultrafiltration was conducted except that the second ultrafiltration treatment was stopped when the electric conductivity of the filtrate became 3.6 mS/cm.

- The protein content of the obtained 0.2% aqueous
20 solution of sodium hyaluronate was 0.09% by weight of sodium hyaluronate, the residue on evaporation was as 320% by weight of sodium hyaluronate and the purity of sodium hyaluronate was 36.7% by weight of the freeze-dried material. Accordingly, the residue on evaporation
25 remained in quantity and the purity of the sodium hyaluronate obtained was low.

CLAIMS:

1. A process for producing an aqueous solution of sodium hyaluronate having high purity, which process comprises treating a fermentation broth of hyaluronic acid with active carbon, optionally after having treated the broth by ultrafiltration, the active carbon-treated broth by ultrafiltration.
2. A process as claimed in claim 1, wherein the active carbon treatment is conducted using a sodium chloride concentration of 0.2 M or more.
3. A process as claimed in claim 1, wherein the ultrafiltration treatment is conducted with an ultrafiltration membrane whose cut-off molecular weight is under hundred thousand, the treated solution being dialyzed to obtain a solution having an electrical conductivity of less than 0.4 mS/cm, and the solution is then concentrated.
4. A process as claimed in claim 1, wherein the purity of sodium hyaluronate is such that it comprises at least 85% by weight of the freeze-dried material obtained by freeze-drying an aqueous solution of sodium hyaluronate, the evaporation residue of the treated solution

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comprises 100 to 130% by weight of sodium hyaluronate and the protein in the treated solution is less than 0.1% by weight of sodium hyaluronate.

5. A process as claimed in claim 1 in which the aqueous solution of sodium hyaluronate contains 1g of sodium hyaluronate per dl of solution.

6. A process as claimed in claim 1 substantially as hereinbefore described with reference to the Examples.

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Relevant Technical fields

- (i) UK CI (Edition K) C3U (UBA, UBD, UCC, UCD, UDE)
- (ii) Int CI (Edition S) C08B

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Databases (see over)

(i) UK Patent Office

(ii) WPI

Date of Search

21 JANUARY 1992

Documents considered relevant following a search in respect of claims

1-6

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
X, Y	GB A 2218429 (CHISSO) page 4 lines 23-28	at least Claim 1
Y	JP A 63141594 (DENKI) abstract	at least Claim 1
Y	JP A 63094988 (MEIJI) abstract	at least Claim 1

SF2(p)

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Category	Identity of document and relevant passages	Relevant to claim(s)

Categories of documents

X: Document indicating lack of novelty or of inventive step.

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